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# Supplementary data

## A direct proofreader–clamp interaction stabilizes the Pol III replicase in the polymerization mode

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## Supplementary materials and methods

### ***Plasmid construction***

Oligonucleotides were purchased from GeneWorks (Adelaide, Australia). Sequences of all PCR-generated inserts used in plasmid construction were confirmed by nucleotide sequence determination, using vector primers PET3 (5'-CGACTCACTATAGGGAGACC-ACAAC) and PET4 (5'-CCTTTCGGGCTTTGTTAGCAG), or other gene-specific primers.

*pSJ1392 ( $\alpha_L$ )*: Plasmid pKO1341 contains *dnaE* between the *NdeI* and *EcoRI* sites of pETMCSI (Neylon *et al*, 2000). It was used as template for primer overlap extension PCR amplification of an internal portion of the *dnaE*[A921L,M923L] gene using the overlapping mutant primers 181 (5'-GCACGCCGAACAGATCCAGCTGACCGATAGCT-

TCC) and 182 (5'-GGAAGCTATCGGTCAGCTGGATCTGTTCGGCGTGC) and outside primers 180 (5'-CCGGAAGAGATGGCTAAGCAACG) and 183 (5'-CATGTCTTTCAGC-CTTACGCCTCC). The PCR product was isolated after digestion with *SexAI* and *StuI* and inserted between the same sites in pKO1341, that had been produced in an *E. coli dcm* strain to yield pSJ1392. This plasmid directs production of the  $\alpha_L$  mutant protein, under control of the phage T7  $\phi 10$  promoter.

*pKO1423* ( $\alpha_{V832G}$ ): The *dnaE*<sup>+</sup> plasmid pND517 (Wijffels *et al*, 2004) was used as template for mutagenesis using 5'-phosphorylated PCR primers 1861 (5'-PO<sub>4</sub>-GACGAC-GGCGAAATCGGCTATGGTATTGGCGCG) and 1862 (5'-PO<sub>4</sub>-GTTGACGTGGAAATG-GTAAAGACCGGAG) and the Phusion site-directed mutagenesis kit (New England BioLabs), to generate pKO1423. This IPTG-inducible *tac* promoter plasmid directs production of the  $\alpha_{V832G}$  protein.

*pKO1430* ( $\alpha\Delta 7$ ): Similar mutagenesis of *dnaE*<sup>+</sup> plasmid pND517 using PCR primers 1879 (5'-PO<sub>4</sub>-TAATACAGGAATACTATGAGTCTGAATTTCTTGA) and 1880 (5'-PO<sub>4</sub>-CTCCGAACCAATGAGGCCACGGAGATCGTTTAATAAAC) and the Phusion kit yielded pKO1430, containing a *dnaE* gene missing the last seven codons (encoding QVELEFD). This plasmid directs overproduction of the  $\alpha\Delta 7$  protein under the transcriptional control of the *tac* promoter.

*pKO1428* ( $\epsilon_{D12A}$ ): The *dnaQ*<sup>+</sup> plasmid pSH1017 (Hamdan *et al*, 2002) was first used as template for site-directed mutagenesis (Phusion kit) to make a plasmid with an *NdeI* site containing the *dnaQ* start codon, using primers 1618 (5'-PO<sub>4</sub>-AGCACTGCAATTACAC-GCCAGATCGTTCTC) and 1619 (5'-PO<sub>4</sub>-CATATGGGGAATTAACCTCCTTAGGATCC-GATTAAAC). This plasmid was used as template to make a further plasmid containing the *dnaQ*[D12A] gene using the same technique and primers 1619 and 1625 (5'-PO<sub>4</sub>-AGCACTGCAATTACACGCCAGATCGTTCTCGCCACCGAAACCACCGG). This plasmid was then digested with *NdeI* and *EcoRI* and the resulting *dnaQ*[D12A] fragment inserted into pETMSCI between the same restriction sites to yield pKO1428. This plasmid directs production the  $\epsilon_{D12A}$  protein under the transcriptional control of the phage T7  $\phi 10$  promoter.

*pKO1429* ( $\epsilon_{D12A,E14A}$ ): Plasmid pKO1428 was used as template for mutagenesis with PCR primers 1874 (5'-PO<sub>4</sub>-ATCGTTCTCGCCACCGCAACCACCGGTATGAAC) and 1875 (5'-PO<sub>4</sub>-CTGGCGTGTAATTGCAGTGCTCATATG) and the Phusion kit, to produce pKO1429. This plasmid directs production of  $\epsilon_{D12A,E14A}$ .

*pSJ1445* ( $\epsilon_Q$ ): Plasmid pSH1017 (Hamdan *et al*, 2002) was used as template for primer overlap PCR amplification of the *dnaQ*[Q182A] gene using overlapping mutagenic primers 406 (5'-CGATGACCGGTGGTGCAACGTCGATGGC) and 407 (5'-GCCATCGACGTTGCACCACCGGTCATCG) and outside vector primers PET3 and PET4. The PCR product was isolated from an agarose gel following digestion with *Bam*HI and *Eco*RI and inserted between the same sites in pETMCSII (Neylon *et al*, 2001) to place the gene under transcriptional control of the phage T7  $\phi$ 10 promoter in plasmid pSJ1445.

*pSJ1446* ( $\epsilon_L$ ): This plasmid, containing the *dnaQ*[T183L,M185L,A186P,F187L] gene was made similarly to pSJ1445, using overlapping primers 408 (5'-GGCGATGACCGGTGGTCAACTGTCGCTGCCGCTGGCGATGGAAGGAGAGACAC) and 409 (5'-GTGTCTCTCCTTCCATCGCCAGCGGCAGCGACAGTTGACCACCGGTCATCGCC); pSJ1446 directs overproduction of the  $\epsilon_L$  protein.

*pSJ1482* (*ubq*- $\epsilon_{CTS}$ ): Plasmid pSH1017 (Hamdan *et al*, 2002) was used for PCR amplification of the 3' portion of *dnaQ* using the forward primer 489 (5'-AAAACCGCGG-TGGTCAAACGTCGATGGCTTTTG), designed to incorporate a *Sac*II site at the codon for Gly180, and reverse primer PET4, preserving an *Eco*RI site just following the stop codon. The isolated PCR product was inserted in-frame between the *Sac*II and *Eco*RI sites following the *His*<sub>6</sub>-*ubq* gene in pKL1426 (Yagi *et al*, 2010) to yield pSJ1482. This T7 promoter vector positions the gene for expression of  $\epsilon_{CTS}$  (C-terminal segment of  $\epsilon$ , residues 181–243) fused to the C-terminus of His<sub>6</sub>-tagged human ubiquitin.

*pCM1503* (*His*<sub>6</sub>- $\beta_{wt}$ ): Plasmid pND262 (Oakley *et al*, 2003) was used as a template for PCR amplification of the *dnaN* gene using primers 414 (5'-A<sub>10</sub>CATATGAAATTTACCGTAGAACGTGAGCATTTATTTAAACCGC), designed to incorporate a *Nde*I site as part of the start codon, and 417 (5'-TTGAATTCTTACAGTCTCATTGGCATGACAACATAAGCC), designed to incorporate an *Eco*RI site just following the TAA stop codon. The PCR product was isolated following digestion with *Nde*I and *Eco*RI and agarose gel electrophoresis and inserted between the same restriction sites in pCL476 (Love *et al*, 1996) to yield plasmid pCM1503. This plasmid directs production of His<sub>6</sub>- $\beta_{wt}$ , with transcription of *dnaN* under control of tandem heat-inducible phage  $\lambda$  *p<sub>R</sub>* and *p<sub>L</sub>* promoters.

*pCM1531* (*His*<sub>6</sub>- $\beta^C$ ): Plasmid pCM1531, which directs expression of His<sub>6</sub>-tagged  $\beta^C$ , a version of the  $\beta$  clamp missing the C-terminal 5 residues (MPMRL) was constructed similarly to pCM1503, using forward primer 562 (5'-T<sub>10</sub>CATATGAAATTTACCGTAGAA-

CGTGAGC and reverse primer 563 (5'-TTTTGAATTCTTAGACAACATAAGCCGCGCT-CTGG).

### **Protein purification**

Buffers were: A, 50 mM Tris.HCl pH 7.6, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol (DTT), 1 mM ATP, 20% (v/v) glycerol; B: 25 mM Tris.HCl pH 7.6, 5 mM DTT, 1 mM EDTA, 20% (v/v) glycerol; C: 50 mM Tris.HCl pH 7.6, 2 mM DTT, 0.5 mM EDTA, 10% (v/v) glycerol; D: 30 mM Tris.HCl pH 7.6, 2 mM DTT, 1 mM EDTA; E: 50 mM Tris-HCl, pH 7.6, 2 mM DTT, 1 mM EDTA; F: 50 mM Tris-HCl, pH 7.6, 5 mM DTT, 1 mM EDTA, 20% (v/v) glycerol; G, 35 mM Tris-HCl, pH 7.6, 0.5 mM DTT, 500 mM NaCl, 15% (v/v) glycerol. All protein purification steps were carried out at 2–6°C.

*Pol III HE subunits:* Overexpression and purification of subunits and complexes that constitute DNA polymerase III HE were described as follows:  $\alpha$ ,  $\delta$  and  $\delta'$  (Wijffels *et al*, 2004);  $\gamma$  and  $\chi$  (Ozawa *et al*, 2005);  $\beta_2$  (Oakley *et al*, 2003);  $\tau$ , refolded  $\psi$  within the  $\chi\psi$  complex,  $\tau\gamma_2\delta\delta'(\pm\psi\chi)$  and  $\tau_3\delta\delta'(\pm\psi\chi)$  complexes (Tanner *et al*, 2008);  $\theta$  (Hamdan *et al*, 2002). The  $\alpha$  variants  $\alpha\Delta 7$ ,  $\alpha_L$  and  $\alpha_{V832G}$  were produced according to methods used for purification of wild-type  $\alpha$ , except that  $\alpha_L$  production was induced in *E. coli* BL21 ( $\lambda$ DE3)/pLysS grown at 30°C with 1 mM IPTG over 3 h.

*The  $\epsilon$  variants* ( $\epsilon_{wt}$ ,  $\epsilon_L$ ,  $\epsilon_Q$ ,  $\epsilon_{D12A}$  and  $\epsilon_{D12A,E14A}$ ) were overproduced in *E. coli* BL21( $\lambda$ DE3)/pLysS strains containing plasmids pSH1017 (Hamdan *et al*, 2002), pSJ1446, pSJ1445, pKO1428, or pKO1429, respectively. Strains were grown individually at room temperature in LB autoinduction medium (Studier, 2005) containing ampicillin and chloramphenicol for 36 h. Cells were lysed using a French press and the pellets containing insoluble proteins were washed and  $\epsilon$  refolded by the methods of Scheuermann and Echols, 1984. Protein(s) were then dialysed in Buffer B and applied onto a 5 ml column of SuperQ-650M resin equilibrated with the same buffer. After the column had been washed with 15 ml of Buffer B,  $\epsilon$  was eluted in a single peak at 70 mM NaCl in a linear gradient (60 ml) of 0–400 mM NaCl in Buffer B.

The *ubq- $\epsilon_{CTS}$*  fusion protein (Figure S1) was overproduced at 37°C in strain BL21 ( $\lambda$ DE3)/pLysS/pSJ1482 in LB medium containing ampicillin and chloramphenicol by addition of IPTG. Lysis, pellet washing and refolding steps were done as for  $\epsilon$ , except that washing steps at 1 M NaCl were omitted. The protein was then purified on a 1 ml HisTrap column (GE Healthcare) according to standard procedure for purification of

His<sub>6</sub>-tagged proteins (Figure S1B).

*Pol III core subassemblies:* Pol III  $\alpha\epsilon\theta$ ,  $\alpha\epsilon_L\theta$ ,  $\alpha\epsilon_Q\theta$ ,  $\alpha\Delta 7\epsilon\theta$  and  $\alpha_L\epsilon\theta$  cores were reconstituted from purified  $\alpha$ ,  $\epsilon$  and  $\theta$  proteins as described (Tanner *et al*, 2008). Protein(s) and complexes were dialysed in Buffer C+70 mM NaCl and applied onto a 5 ml column of SuperQ-650M resin equilibrated with the same buffer. After the column had been washed with 20 ml of Buffer C+70 mM NaCl to remove excess of  $\epsilon$ ,  $\theta$ , and  $\epsilon\theta$ , pure core was eluted in a single peak at 150 mM NaCl in a linear gradient (20 ml) of 70–1600 mM NaCl in Buffer C.

*The  $\gamma$  complex:* The alternative clamp loader  $\gamma_3\delta\delta'$  (non-proficient in assembling DNA Pol III HE) was made by mixing  $\gamma$  with equimolar excess of  $\delta$  and  $\delta'$ , thereby ensuring excess  $\delta$  remained in complex with  $\delta'$  ( $\delta\delta'$  complex) to prevent precipitation due to the insolubility of  $\delta$ . The mixture was dialysed in Buffer D+50 mM NaCl and applied onto a 5 ml column of Q-Sepharose resin (GE Healthcare) equilibrated with the same buffer. After the column had been washed with 10 ml of Buffer D+50 mM NaCl, pure  $\gamma_3\delta\delta'$  complex was eluted in a single peak at 270 mM NaCl in a linear gradient (190 ml) of 50–450 mM NaCl in Buffer D. The peak was resolved from that containing excess  $\delta\delta'$  complex that eluted at 170 mM NaCl.

*Sliding clamps:* (His<sub>6</sub>- $\beta_{wt}$ )<sub>2</sub> was purified according to procedure for purification of  $\beta_2$  (Oakley *et al*, 2003) except that one of the chromatographic steps using a DEAE-Fractogel column was substituted by purification on a 1 ml HisTrap column. In contrast to its wild-type counterpart, overproduced (His<sub>6</sub>- $\beta^C$ )<sub>2</sub> was insoluble. Following lysis in Buffer E, the insoluble pellet was washed twice in Buffer E+1 M NaCl and then twice in Buffer E (without NaCl). The pellet was then resuspended in Buffer F until homogeneous. Proteins were unfolded in denaturing Buffer F+3.5 M guanidine.HCl (Gu.HCl) and after addition of protease inhibitor cocktail C (Roche) according to the manufacturer's prescription, made up to the composition of Buffer F+500 mM NaCl+1 M GuHCl and refolded by dialysis against two changes of Buffer F+500 mM NaCl. After centrifugation (35000 × g, 40 min), the supernatant containing refolded (His<sub>6</sub>- $\beta^C$ )<sub>2</sub> was dialysed against two changes of Buffer G+35 mM imidazole and again clarified by centrifugation (35000 × g, 40 min). The supernatant was applied to a 1 ml HisTrap column that had been equilibrated in the same buffer. After the column had been washed with 15 ml of Buffer G, (His<sub>6</sub>- $\beta^C$ )<sub>2</sub> was eluted at 170 mM imidazole in a linear gradient (10 ml) of 35–500 mM imidazole in Buffer G. The monomeric molecular mass of

the His<sub>6</sub>-β<sup>C</sup> measured by nanoESI-MS after dialysis into 0.1% (v/v) formic acid was 40911 Da, corresponding well with the calculated mass of 40911.7 Da.

To minimize undesired subunit exchange when preparing the His<sub>6</sub>-β<sub>wt</sub>/β<sub>wt</sub> and His<sub>6</sub>-β<sup>C</sup>/β<sub>wt</sub> heterodimers, all purification and dialysis steps (subsequent to the initial exchange) were carried out at 4°C and as rapidly as possible. Samples of each purified mixed dimer were dialysed into 200 mM ammonium acetate, pH 7.2, and analysed by nanoESI-MS (Figure S2).

The His<sub>6</sub>-β<sub>wt</sub>/β<sub>wt</sub> heterodimer was prepared by mixing an 8-fold molar excess of (β<sub>wt</sub>)<sub>2</sub> with (His<sub>6</sub>-β<sub>wt</sub>)<sub>2</sub>, and the mixture was dialysed against 3 changes of 2 l of Buffer SE1 (30 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 M NaCl, 10 mM MgCl<sub>2</sub>) containing 10% (v/v) glycerol for 12 h at 4°C to allow subunit exchange, before being dialysed against 3 changes of 2 l of isolation buffer (30 mM Tris-HCl, pH 7.6, 30 mM NaCl, 0.5 mM dithiothreitol, 10% v/v glycerol, 30 mM imidazole) for a further 12 h. The sample was loaded onto a 1 ml Ni<sup>2+</sup>-charged HisTrap column (GE Healthcare) that had been equilibrated in the same buffer, then washed with 10 ml of the same buffer before being eluted with a linear gradient (10 ml) of 30–500 mM imidazole in isolation buffer. Fractions containing His<sub>6</sub>-β<sub>wt</sub>/β<sub>wt</sub>, which eluted at about 110 mM imidazole, were immediately pooled and dialysed into 30 mM Tris-HCl, pH 7.6, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 30% (v/v) glycerol, giving a final yield of ~1 mg of His<sub>6</sub>-β<sub>wt</sub>/β<sub>wt</sub> in a total volume of 0.9 ml (13.4 μM). Aliquots were stored frozen at –80°C until immediately before use.

To prepare the His<sub>6</sub>-β<sup>C</sup>/β<sub>wt</sub> hemi-mutant heterodimer, a 15-fold molar excess of (β<sub>wt</sub>)<sub>2</sub> was added to (His<sub>6</sub>-β<sup>C</sup>)<sub>2</sub>, and the mixture was dialysed against 3 changes of 2 l of Buffer SE1 for a total of 12 h at 4°C, before being dialysed for a further 12 h against 3 changes of 2 l of isolation buffer (as above but containing 40 mM imidazole). The sample was loaded onto and eluted from a 1 ml Ni<sup>2+</sup>-charged HisTrap column as described above for the His<sub>6</sub>-β<sub>wt</sub>/β<sub>wt</sub> dimer. Fractions containing the His<sub>6</sub>-β<sup>C</sup>/β<sub>wt</sub> hemi-mutant, which eluted at ~100 mM imidazole, were immediately pooled and dialysed into 30 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 30% (v/v) glycerol, yielding ~0.57 mg of His<sub>6</sub>-β<sup>C</sup>/β<sub>wt</sub> in a volume of 3 ml (~2.3 μM). Aliquots were frozen in liquid nitrogen and stored at –80°C until use.

**DnaBC complex:** The DnaB helicase and DnaC helicase loader were as described in San Martin *et al* (1995). The DnaBC complex was made by mixing DnaB (11 mg) with

excess DnaC (10 mg) in 38 ml of Buffer A+100 mM NaCl+1 mM ATP and applied to a 5 ml column of SuperQ-650M resin (Toyopearl) equilibrated in the same buffer. After the column had been washed with 20 ml of Buffer A+100 mM NaCl to remove excess DnaC, DnaBC was eluted in a single peak in a linear gradient (60 ml) of 100–800 mM NaCl in Buffer A.

*PriA*, *PriB* and *DnaT* were prepared from overproducing strains by methods similar to those described by Marians (1995).

### ***DNA replication assays***

*Preparation of 5'-flap-primed ssM13 DNA template:* Wild-type phage M13 was purified from infected culture supernatants by PEG precipitation and banding (twice) in CsCl gradients. Phage were lysed, phenol extracted, and the ssDNA precipitated with ethanol, resuspended in and dialysed extensively against 10 mM Tris.HCl pH 8.0, 1 mM EDTA (TE buffer) and stored frozen at  $-80^{\circ}\text{C}$ . Oligonucleotide primer 48 has the sequence 5'-T<sub>36</sub>TATGTACCCCGGTTGATAATCAGAAAAGCCCCA-3', consisting of a 33-mer complementary to wild-type M13 DNA preceded at the 5' end by a non-complementary (dT)<sub>36</sub> flap. M13 ssDNA (35 nM, as circles) was mixed with 1  $\mu\text{M}$  of primer 48 in 30 mM Tris.HCl pH 7.6, 15 mM MgCl<sub>2</sub>, 130 mM NaCl and 0.1 mM EDTA. The mixture was treated at  $55^{\circ}\text{C}$  for 10 min and then cooled slowly to room temperature over a period of 8 h.

*Preparation of TFII DNA template:* The 5'-flap-primed ssM13 DNA template was pre-filled in the standard SD reaction (see below; total volume 420  $\mu\text{l}$ ) but containing 14 nM DNA template and in the absence of SSB to prevent the Pol III SD reaction for 20 min. Products were separated on a 0.7% agarose gel in 2xTBE (180 mM Tris-borate, 4 mM EDTA) running buffer containing 1  $\mu\text{g/ml}$  EtBr. Gel bands containing TFII were excised and the DNA isolated by electroelution into a dialysis sac (Sambrook and Russell, 2001), extensively dialysed in TE buffer, concentrated to 1 ml using an Amicon Ultra-15 centrifugal filter device and then to 120  $\mu\text{l}$  using a QIAEX II Agarose Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The final concentration in TE buffer was 35 nM TFII DNA.

*Coupled Pol III primer extension-strand displacement (SD) rolling circle assay:* The standard SD replication reaction contained: 2.5 nM 5'-flap-primed M13 ssDNA template, 1 mM ATP, 0.5 mM of each dNTP, 30 nM  $\tau_3\delta\delta'\psi\chi$  clamp loader, 90 nM  $\alpha\epsilon\theta$  core, 200



nM  $\beta_2$  clamp and 750 nM SSB in buffer comprised of 25 mM Tris.HCl pH 7.6, 10 mM  $\text{MgCl}_2$ , 10 mM DTT and 130 mM NaCl, in a final volume of 13  $\mu\text{L}$ . When pre-assembled  $\alpha\epsilon_Q\theta$  or  $\alpha\epsilon_L\theta$  cores or  $\text{His}_6\text{-}\beta_{\text{wt}}/\beta_{\text{wt}}$ ,  $\text{His}_6\text{-}\beta^{\text{C}}/\beta_{\text{wt}}$  or  $(\text{His}_6\text{-}\beta^{\text{C}})_2$  clamps were used, their concentrations were the same as of appropriate counterparts in the standard reaction. Assays containing various core sub-complexes assembled *in situ* contained 100 nM  $\alpha$  or  $\alpha_{\text{V832G}}$  + 350 nM  $\epsilon$ ,  $\epsilon_{\text{D12A}}$ ,  $\epsilon_{\text{D12A,E14A}}$  or  $\text{ubq-}\epsilon_{\text{CTS}} \pm 1 \mu\text{M } \theta$ . To confirm the indispensability of the  $\psi\chi$  complex in SD synthesis, the standard assay contained 30 nM  $\tau_3\delta\delta'$  or  $\tau_3\delta\delta'\psi\chi$  clamp loader complex, the latter assembled *in situ* with 30 nM  $\tau_3\delta\delta'$  and 110 nM  $\psi\chi$ . Likewise, the requirement for multiple  $\tau$  subunits in Pol III SD synthesis was interrogated in standard reactions that had the  $\tau_3\delta\delta'\psi\chi$  clamp loader substituted by one containing 50 nM  $\tau_2\delta\delta'\psi\chi$  (one  $\tau$  subunit) or with clamp loader assembled *in situ* from 50 nM  $\gamma_3\delta\delta'$  and 110 nM  $\psi\chi$  (no  $\tau$  subunit). The requirement of the  $\chi$ -SSB interaction for SD was assessed in the standard assay containing 750 nM  $\text{SSB}\Delta 8$  (Mason *et al*, 2013) in place of SSB.

*Pol III strand displacement (SD) rolling circle assay:* This was carried out in the standard reaction conditions except that the isolated TFII DNA template was used instead of the 5'-flap-primed ssM13 DNA template.

*Assays of primer extension, under “difficult conditions”:* Assays generally contained 2.5 nM 5'-flap-primed ssM13 DNA template, 1 mM ATP, 0.5 mM of each dNTP, 10 mM DTT, 40 nM  $\gamma_3\delta\delta'$  clamp loader, 150 nM  $\alpha\epsilon\theta$  core and 200 nM  $\beta_2$  in 25 mM Tris.HCl pH 7.6, 10 mM  $\text{MgCl}_2$  and 130 mM NaCl in final volume of 13  $\mu\text{L}$ . Particular reactions contained wild type core substituted by  $\alpha$ , or isolated  $\alpha\epsilon_L\theta$ ,  $\alpha\epsilon_Q\theta$ ,  $\alpha\Delta 7\epsilon\theta$  or  $\alpha_L\epsilon\theta$  complexes, each at 150 nM.

Replication components (except DNA) were mixed and treated for 5 min at room temperature to allow proteins to interact, cooled in ice and DNA added. The reactions were initiated by quick transfer to a 30°C water bath, and quenched at indicated times by addition to 11  $\mu\text{L}$  of 200 mM EDTA pH 8.0, 0.08% (w/v) bromophenol blue, 0.08% (w/v) xylene cyanol, 10% (v/v) glycerol, 2% (w/v) SDS. Reaction mixtures were treated for 2 min at 42°C, loaded onto a 0.8% agarose gel in 2×TBE buffer and electrophoresis carried out at 45 V for 180 min. Each gel was stained in 200 ml of SYBR<sup>®</sup> gold nucleic acid stain (Tuma *et al*, 1999) at the concentration suggested by the supplier (Invitrogen, Carlsbad, CA). DNA bands were visualized under 302-nm UV light using a Gel Doc<sup>™</sup> XR+ system (Bio-Rad, Hercules, CA). A corresponding DNA template sample was

loaded in one lane of each gel as a reference, as well as a sample of GeneRuler™ 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

### ***Single molecule leading strand replication assays***

Continuous flow leading strand single molecule DNA replication assays were carried out essentially as described previously (Tanner *et al*, 2008; Tanner and van Oijen, 2009), with a few modifications concerning general and specific protein content and experimental conditions as described below. We also replaced the 24-mer primer on the leading strand at the fork with a 30-mer. Replication proteins were introduced in SM replication buffer [50 mM HEPES.KOH pH 7.9, 80 mM KCl, 12 mM Mg(acetate)<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 195 μM of each dNTP and were present continuously in the flow calibrated to give a force of ~3 pN during the course of experiments at the following concentrations: 60 nM core polymerase ( $\alpha$  or isolated  $\alpha_{\epsilon\theta}$ ,  $\alpha_{\epsilon_L\theta}$  or  $\alpha_{\epsilon_Q\theta}$ ), 30 nM  $\tau_3\delta\delta'\chi\psi$  clamp loader, 30 nM  $\beta_2$  clamp, 30 nM isolated DnaB<sub>6</sub>(DnaC)<sub>6</sub> helicase/loader complex, and the fork restart proteins PriA at 20 nM, PriB at 40 nM and DnaT at 480 nM. Experiments were done at 32–34°C. Data were acquired and treated as before except that pauses were selected as a minimum of six data points (images taken at 2 Hz) with amplitude fluctuations less than three times the standard deviations of the noise.

### ***Analysis of $\beta_2$ - $\epsilon$ and $\alpha$ -ubq- $\epsilon_{CTS}$ interactions by gel filtration chromatography***

Analytical gel filtration was carried out at 4°C using an Äkta FPLC system (GE Healthcare). Buffers were: GF1, 50 mM Tris.HCl pH 7.6, 150 mM NaCl, 2 mM DTT, 0.5 mM EDTA and 20% glycerol; GF2, 30 mM Tris.HCl pH 7.6, 150 mM NaCl, 2 mM DTT, 0.5 mM EDTA and 10% glycerol.

Analysis of  $\beta_2$ - $\epsilon$  interactions (Figure S3B) was carried out using mixtures of 17 μM  $\beta_2$  and 57 μM  $\epsilon$ ,  $\epsilon_Q$  or  $\epsilon_L$  in 300 μl of Buffer GF1, treated overnight at 4°C prior to loading onto and elution at 0.3 ml/min from a column (1 × 40 cm) of Sephacryl S-200 HR resin (GE Healthcare) equilibrated in Buffer GF1. Fractions of 500 μL were collected, and proteins in a portion (120 μl) of each were precipitated with ice cold acetone (700 μl). After 15 min on ice, protein pellets were collected by centrifugation (30000 × g; 60 min), dried for 10 min in air, dissolved in 25 μl of loading buffer and separated in 4–12% gradient SDS-PAGE gels (Invitrogen). Gels were stained with Coomassie blue.

Analysis of the interaction between  $\alpha$  and ubq- $\epsilon_{CTS}$  (Figure S1C) was carried out similarly using a mixture of 7  $\mu$ M  $\alpha$  and 16  $\mu$ M ubq- $\epsilon_{CTS}$  in 300  $\mu$ l of Buffer GF2. Protein complexes were resolved on a column (1  $\times$  40 cm) of Sephacryl S-100 resin (GE Healthcare), and analysed similarly, except that all of the collected fractions (500  $\mu$ l) were treated with acetone in preparation for analysis by SDS-PAGE.

### ***Analysis of $\epsilon_{pep}$ - $\beta_2$ interactions by multiplex SPR technology***

A 6  $\times$  6 multiplex ProteOn XPR-36 Protein Interaction Array System was used for binding studies, carried out at 20°C using the ProteOn™ NLC sensor chip with a NeutrAvidin-coated surface for immobilization of biotinylated ligands. The N-terminally biotinylated  $\epsilon$  peptide ligands had sequences as follows:  $\epsilon_{Lpep}$ , bio-linker-GGQLSLPLAV (98% purity),  $\epsilon_{WTpep}$ , bio-linker-GGQTSMFAV (86%), and  $\epsilon_{Qpep}$ , bio-linker-GGATSMFAV (81%). Peptides were designed to incorporate the amino acid sequences (residues 182–187 in  $\epsilon$ ) proposed to interact with  $\beta_2$  in the middle of the decapeptide sequences. The two glycines at the N-terminus and the penultimate alanine are native to  $\epsilon$  while the native methionine was substituted by valine at the C-terminus to improve peptide solubility as suggested by the supplier (Mimotopes Pty Ltd). An  $\epsilon$ -aminohexanoic acid linker was incorporated to distance protein-interacting residues from the surface of the sensor chip, onto which the peptide is immobilized. Peptides were dissolved to 1 mM in 100 mM Tris.HCl pH 7.6, 25% (v/v) acetonitrile. Purity of the peptides does not influence binding studies since only the authentic products contain biotin.

All 36 interaction spots of the sensor chip were activated with three sequential injections of 1 M NaCl, 50 mM NaOH across six vertical (ligand) flow paths (40 s each at a flow rate of 40  $\mu$ l/min) and six horizontal (analyte) flow paths (40 s each at 100  $\mu$ l/min). The surface was further stabilized by two injections of 1 M MgCl<sub>2</sub> in each direction, and with the same contact times and flow rates. Solutions of 100 nM  $\epsilon_{Lpep}$ ,  $\epsilon_{WTpep}$  and  $\epsilon_{Qpep}$  in SPR buffer [50 mM Tris.HCl pH 7.6, 50 mM NaCl, 0.5 mM tris(carboxyethyl)phosphine, 0.2 mM EDTA, 0.005% surfactant P20 (GE Healthcare)] were injected for 33 s at a flow rate of 50  $\mu$ l/min to immobilize each peptide onto a discrete ligand channel, to obtain responses of ~50, 70 and 60 RU, respectively, across 6 interaction spots. The chip was then rotated 90° and binding studies carried out by sequential injection of three different serially-diluted concentration series of  $\beta_2$  in SPR buffer (a, 0–0.098; b, 0–3.12; and c, 0–100  $\mu$ M), in the analyte direction at 100  $\mu$ l/min for 60 s followed by dissociation in SPR

buffer over 300 s. Based on similar responses upon sequential injections of  $\beta_2$  at the same concentration over the immobilized  $\beta$ -binding peptide, as well as efficient and complete dissociation of the analyte, we confirmed the previous observation (Wijffels et al., 2004) that regeneration steps in this assay are unnecessary. The final sensorgrams were generated by double (two dimensional) reference subtraction (subtraction of the responses from corresponding interaction spots of a non-modified ligand flow path in one dimension and with  $[\beta_2] = 0$  in the second. Concentration series a and b were used for determination of  $K_D(\epsilon_{Lpep}-\beta_2)$  while the series c was used to determine  $K_D(\epsilon_{WTpep}-\beta_2)$  and  $K_D(\epsilon_{Qpep}-\beta_2)$ .

Considering that fast on/fast off kinetics were observed, equilibrium responses ( $R$ ) at various concentrations of  $\beta_2$  were used to fit binding isotherms to obtain  $K_D$  and  $R_{max}$  (response at saturation of ligand binding sites), using KaleidaGraph (Synergy software); for 1:1 binding,  $R/R_{max} = [\beta_2] / (K_D + [\beta_2])$  [eq. 1]. Due to low responses (extremely weak binding) in the case of the  $\epsilon_{Qpep}-\beta_2$  interaction, the  $R_{max}$  value was estimated based on values for the other two interactions and relative ratios of immobilized peptides, and the measured  $R$  with  $\beta_2$  at 50  $\mu$ M was used in eq. 1 to estimate  $K_D(\epsilon_{Qpep}-\beta_2)$ .

### **Assessment of $\epsilon$ - $\beta_2$ interactions by ESI-MS**

Protein samples for nanoESI-MS studies were prepared by dialysis against 5 changes of ammonium acetate (140–500 mM, as specified), pH 7.6, containing 1 mM  $\beta$ -mercaptoethanol for a minimum 4 h per buffer change. Mass spectra for study of interactions of  $\epsilon$  variants or purified  $\alpha\epsilon\theta$  containing  $\epsilon$  variants with  $\beta_2$  (at indicated concentrations) were acquired in positive ion mode using nanoelectrospray ionization (nanoESI) on a Waters (Wythenshawe, UK) extended mass range Q-ToF Ultima™ mass spectrometer fitted with a Z-spray ESI source. Mass spectra were obtained using the following parameters: capillary voltage 1.5 kV, cone voltage 120 V, RF lens 1200 V, transport and aperture 5 V and collision energy 2 V. The pressure in the ion optics region was set to 0.1 mbar. Likewise, mass spectra showing interactions between  $\beta_2$  and *in-situ* assembled  $\alpha\epsilon\theta$ , including  $\alpha$  and  $\epsilon$  variants (at indicated concentrations) were acquired in positive ion mode using nanoESI on a Waters Synapt™ HDMS mass spectrometer fitted with a Z-spray ESI source. Parameters were: capillary voltage 1.5 kV, cone voltage 150 V, extraction cone voltage 6 V, trap collision energy 10 V and transfer collision energy 6 V. The backing pressure was set to 6.5 mbar. Both instruments were calibrated using 10 mg/ml cesium iodide in 70% isopropanol/water. In

each study, 100–150 acquisitions were combined and the resulting spectrum was baseline subtracted and smoothed using the Savitzky-Golay algorithm. Spectra were acquired over a  $m/z$  range of 500–15000 and analyzed using MassLynx™ software. For illustrative purposes, only the relevant  $m/z$  range of each spectrum has been presented in the Figures.

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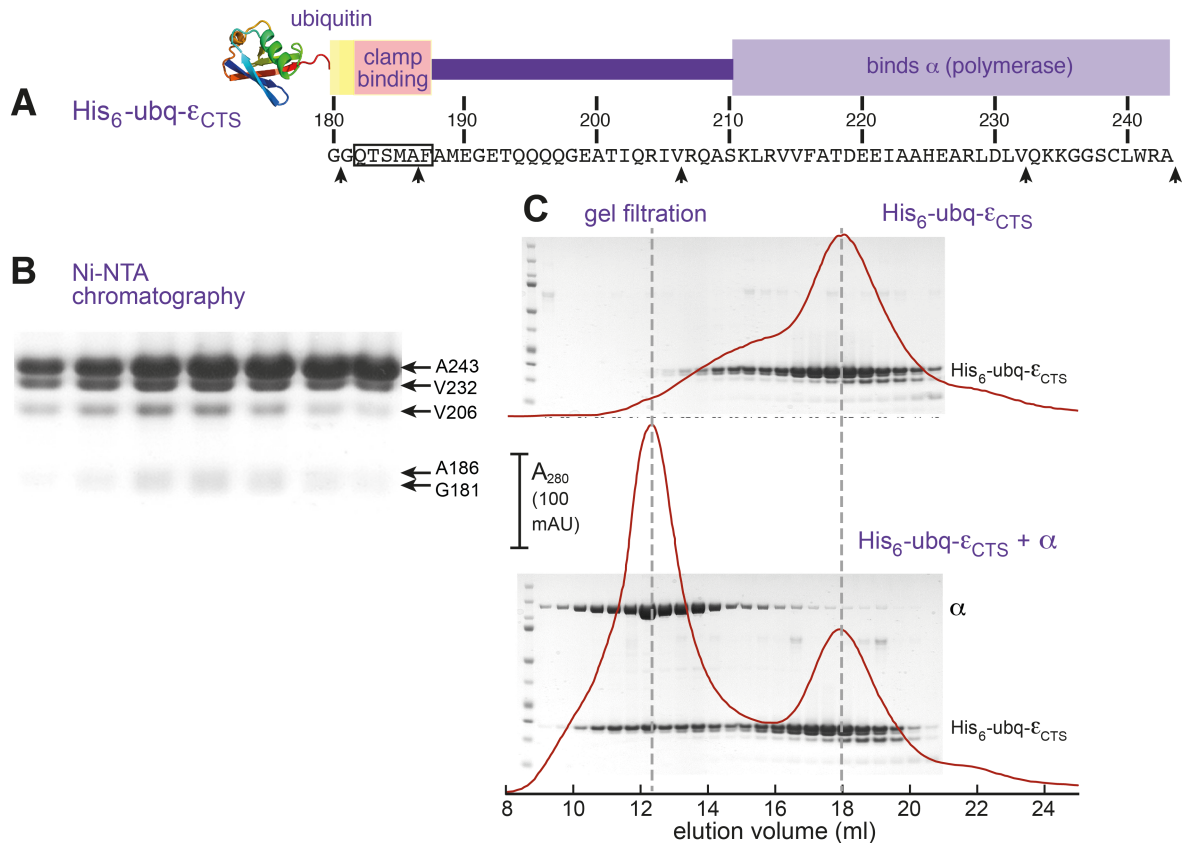
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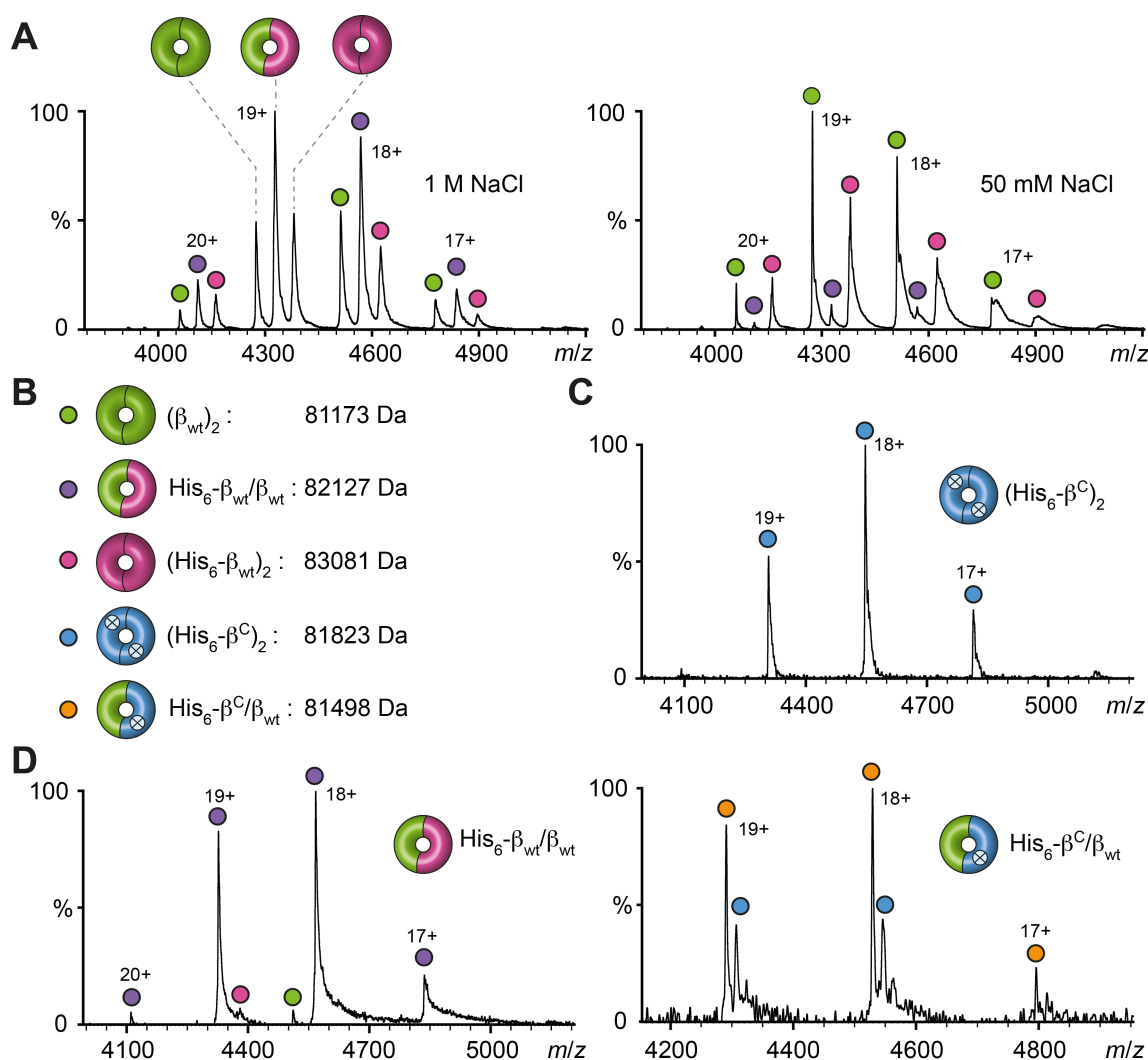
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## Supplementary figures:

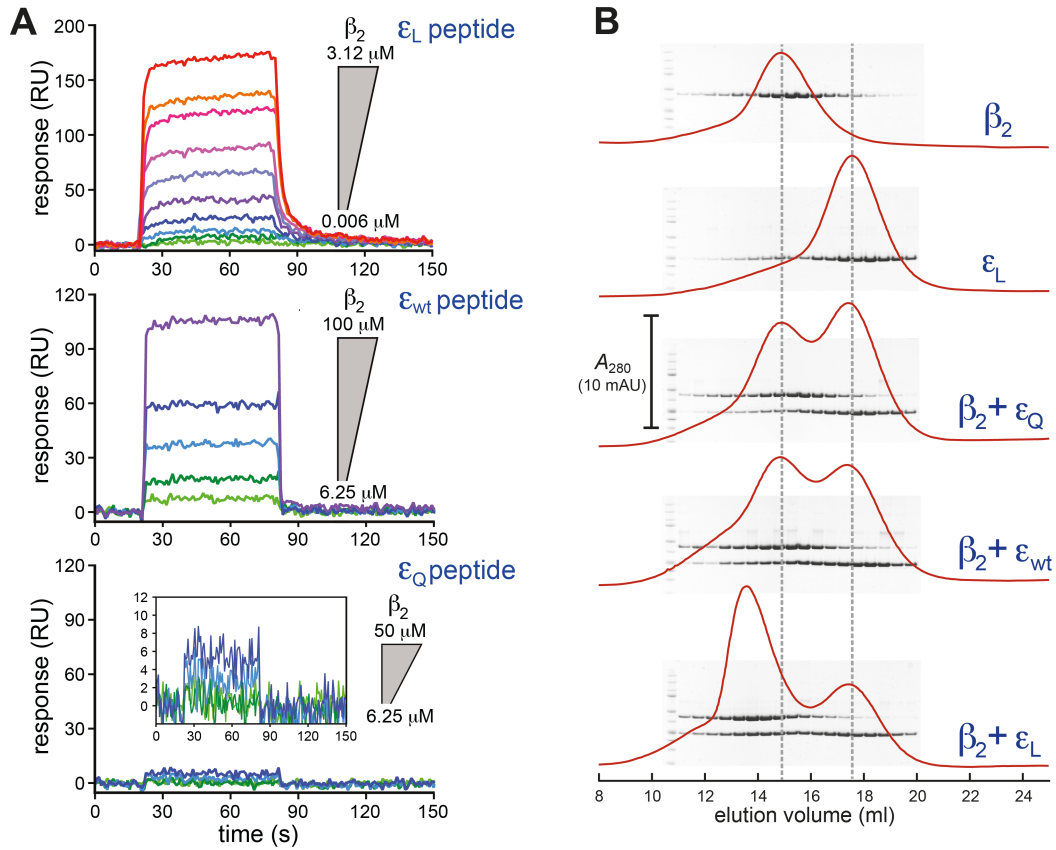


**Figure S1** Ubq-ε<sub>CTS</sub>, an ε derivative that lacks the complete exonuclease domain, forms a stable complex with α. **(A)** Two-domain organization of ubq-ε<sub>CTS</sub>. The flexible ε<sub>CTS</sub> (residue numbering based on the *E. coli* ε sequence and shown in single letter code) was fused to human ubiquitin (79 residues) via the common Gly–Gly residue pair at the C-terminus of both ubiquitin and the N-terminal exonuclease domain of ε. Arrows in ε<sub>CTS</sub> indicate sites of proteolytic cleavage identified in Figure S1B. **(B)** Sites of cleavage by unidentified *E. coli* protease(s) in ubq-ε<sub>CTS</sub>. SDS-PAGE analysis of successive fractions collected during elution of His<sub>6</sub>-ubq-ε<sub>CTS</sub> from a Ni-NTA column. ESI-MS in 0.1% (v/v) formic acid was used to identify full-length His<sub>6</sub>-ubq-ε<sub>CTS</sub> and N-terminal proteolytic fragments, where cleavage occurs at the C-terminal side of the indicated residues. **(C)** Full-length His<sub>6</sub>-ubq-ε<sub>CTS</sub> forms a stable complex with α, isolable by gel filtration. Lower panel: The complex formed by mixing α (7 μM) and His<sub>6</sub>-ubq-ε<sub>CTS</sub> (16 μM) was resolved from excess His<sub>6</sub>-ubq-ε<sub>CTS</sub> and its proteolysis products using a column (1 x 40 cm) of Sephacryl S-100 HR. Upper panel: For comparison, the same quantity of His<sub>6</sub>-ubq-ε<sub>CTS</sub> was chromatographed on the same column. Samples from corresponding peak fractions were analysed on a 4–12% SDS-PAGE gel, stained with Coomassie blue.

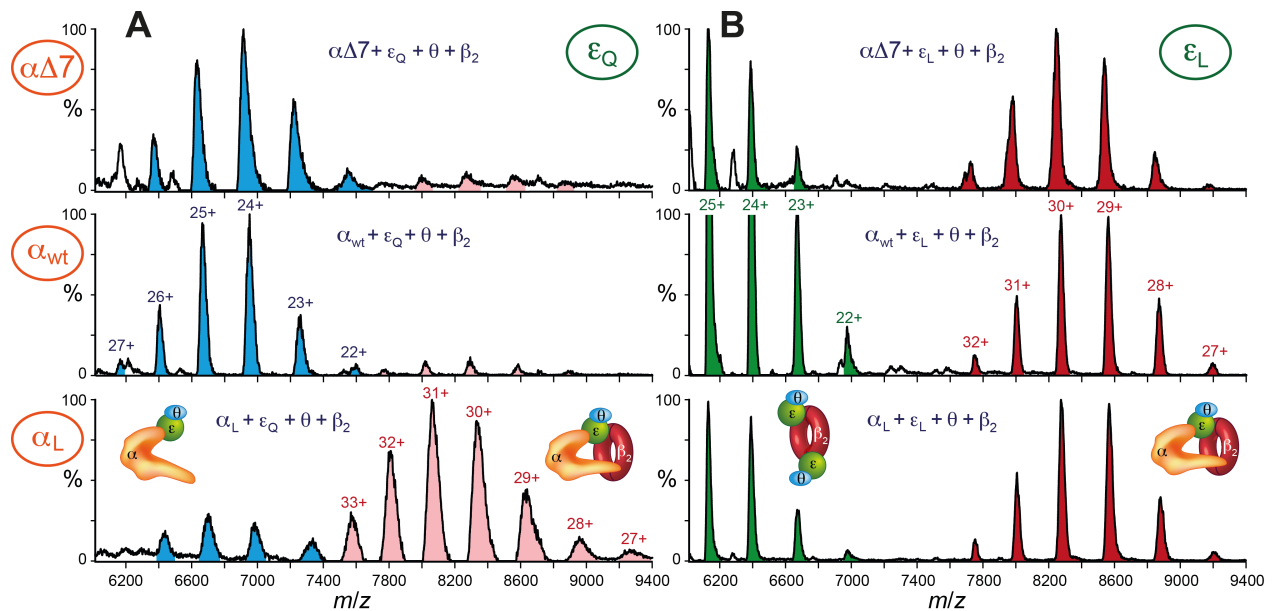


**Figure S2** Isolation of mixed  $\beta$ -dimers with both, one or no protein binding sites occluded. **(A)** Optimization of conditions for isolation of  $\beta_2$  heterodimers; high salt concentration promotes subunit exchange while low salt conditions stabilize the dimer. Equimolar mixtures (2 or 3  $\mu\text{M}$  each) of  $(\beta_{wt})_2$  and  $(\text{His}_6\text{-}\beta_{wt})_2$  in 30 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, containing either 10 mM  $\text{MgCl}_2$  and 1 M NaCl (left panel) or 50 mM NaCl and 10% glycerol (right panel) were placed at 4°C for 12 h, then dialysed against 200 mM ammonium acetate, pH 7.4 before analysis by positive ion nanoESI-MS. Assuming that  $(\beta_{wt})_2$ ,  $(\text{His}_6\text{-}\beta_{wt})_2$  and  $\text{His}_6\text{-}\beta_{wt}/\beta_{wt}$  species ionize with similar efficiencies, almost complete exchange is observed in 1 M NaCl and very little in low salt buffers. **(B)** Schematic representation of various homo- and hetero-dimeric sliding clamp species. The non-functional protein binding sites on the  $\beta^C$  mutant subunits are denoted with  $\otimes$ . **(C)**  $(\text{His}_6\text{-}\beta^C)_2$  is a stable dimer. Positive ion nanoESI mass spectrum of  $(\text{His}_6\text{-}\beta^C)_2$  in 200 mM ammonium acetate, pH 7.2. **(D)** NanoESI-MS of purified  $\beta$  heterodimers produced by subunit exchange.  $\text{His}_6\text{-}\beta_{wt}/\beta_{wt}$  (left panel) and  $\text{His}_6\text{-}\beta^C/\beta_{wt}$  (right panel) were dialysed into 200 mM ammonium acetate, pH 7.2 before analysis. The  $\text{His}_6\text{-}\beta^C/\beta_{wt}$  that has one binding site in the dimer occluded is contaminated by a some  $(\text{His}_6\text{-}\beta^C)_2$ . However, this construct that has both protein binding sites occluded is non-functional in Pol III-dependent DNA replication (Figure 2E).

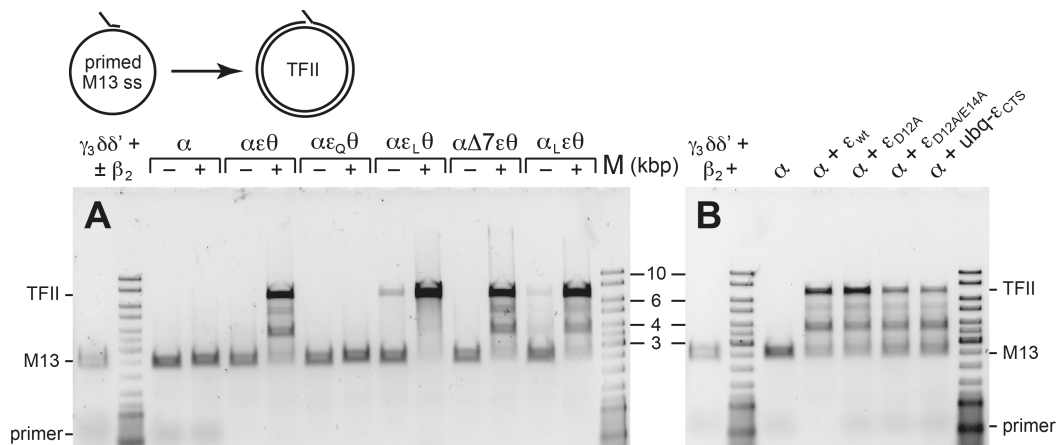




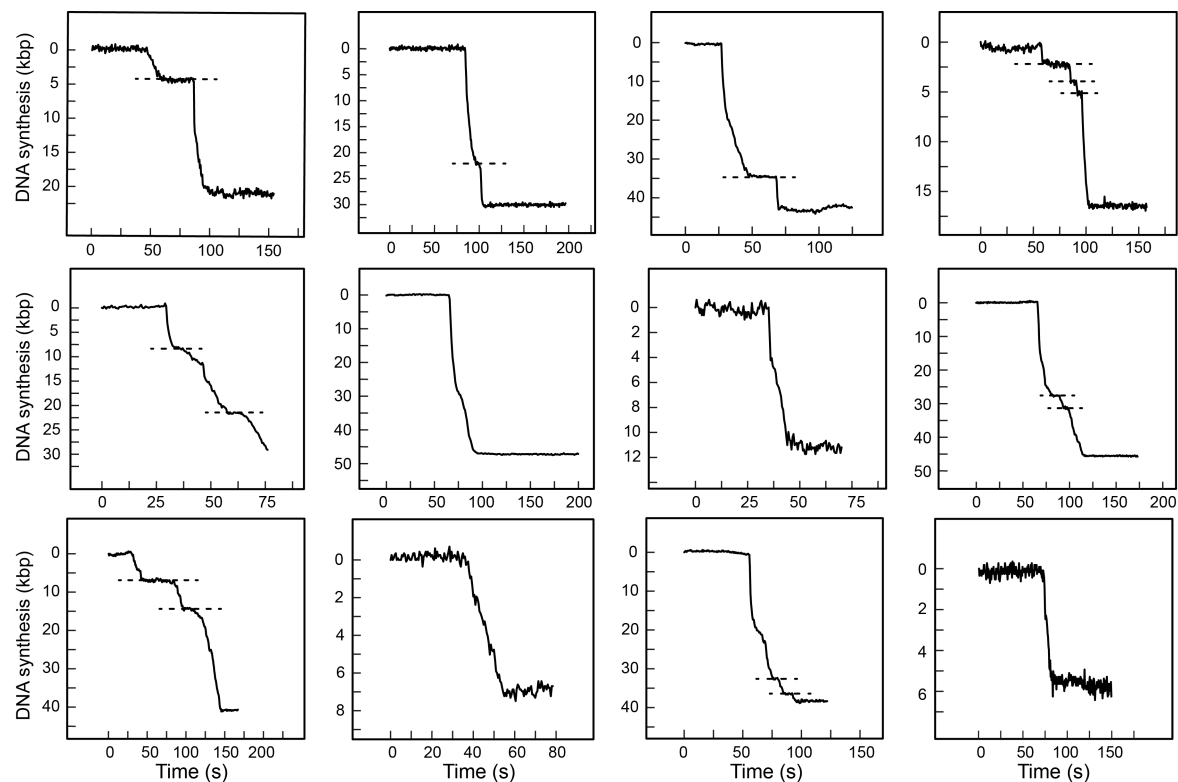
**Figure S3** Physical evidence for interaction of  $\beta_2$  with the CBM in  $\epsilon$ . **(A)** SPR sensorgrams showing binding of  $\beta_2$  to immobilized  $\epsilon_L$ ,  $\epsilon_{WT}$  and  $\epsilon_Q$  peptides, as indicated. Binding studies were carried out by monitoring responses during 60 s of injection of  $\beta_2$  samples in concentration series made by twofold serial dilution (the highest and the lowest concentration in each series are indicated) followed by 300 s dissociation. For all immobilized peptides, binding of  $\beta_2$  has fast on/fast off kinetics. Responses measured at equilibrium were fit to equation 1 (Supplementary methods) to generate binding isotherms (Figure 3A) for  $\beta_2$ - $\epsilon_{L\text{pep}}$  ( $K_D = 0.38 \pm 0.04 \mu\text{M}$ ;  $R_{\text{max}} = 184 \pm 6 \text{ RU}$ ) and  $\beta_2$ - $\epsilon_{WT\text{pep}}$  ( $K_D = 210 \pm 50 \mu\text{M}$ ;  $R_{\text{max}} = 327 \pm 56 \text{ RU}$ ) interactions. Due to the extremely weak response, we were unable to fit a binding isotherm for the  $\beta_2$ - $\epsilon_{Q\text{pep}}$  interaction but instead calculated an approximate value of  $K_D$ . Based on fit  $R_{\text{max}}$  values for  $\beta_2$ - $\epsilon_{L\text{pep}}$  and  $\beta_2$ - $\epsilon_{WT\text{pep}}$  interactions and measured responses upon immobilization of  $\epsilon_{L\text{pep}}$ ,  $\epsilon_{WT\text{pep}}$  and  $\epsilon_{Q\text{pep}}$  of 50, 70 and 60 RUs respectively, the  $R_{\text{max}}$  value for  $\beta_2$ - $\epsilon_{Q\text{pep}}$  interaction was  $\sim 260 \text{ RU}$ . Using the measured  $\sim 5 \text{ RU}$  response resulting from injection of  $50 \mu\text{M}$   $\beta_2$  and this estimated  $R_{\text{max}}$ , equation 1 was used to calculate  $K_D$  ( $\beta_2$ - $\epsilon_{Q\text{pep}}$ )  $\sim 2.5 \text{ mM}$ . **(B)** Although only few residues away from the structured exonuclease domain (Figure 1A), the CBM in  $\epsilon_L$  is accessible to the  $\beta_2$  dimer. Protein mixtures (300  $\mu\text{L}$ ) of  $\beta_2$  (17  $\mu\text{M}$ ) and  $\epsilon_Q$ ,  $\epsilon_{WT}$  or  $\epsilon_L$  (57  $\mu\text{M}$  each), and  $\epsilon_L$  or  $\beta_2$  alone, as indicated, were gel filtered through a Sephacryl S-200 HR column (1 x 40 cm). Samples from peak fractions were analysed on a 4–12% SDS-PAGE gel, stained with Coomassie blue. The bottom panel shows clear evidence of interaction of  $\epsilon_L$  with  $\beta_2$ .



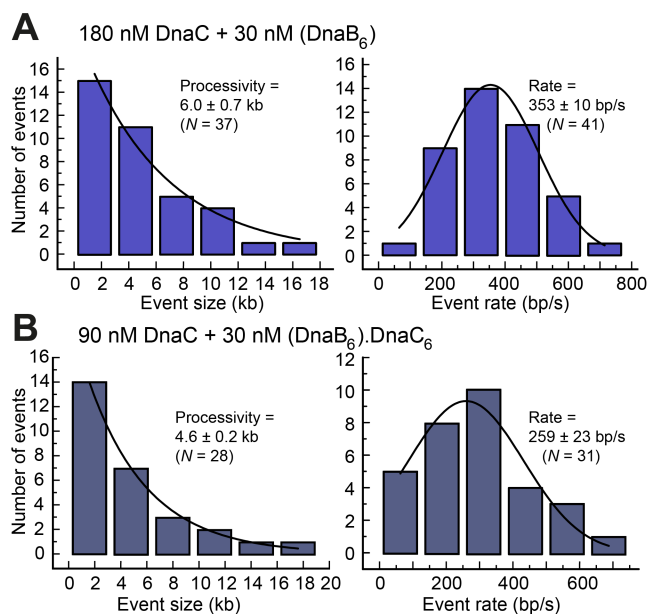
**Figure S4** Simultaneous interaction of the CBM in  $\epsilon$  and the internal CBM in  $\alpha$  with  $\beta_2$  stabilizes the  $\alpha\epsilon\theta$ - $\beta_2$  Complex. Strengthening of the CBMs in either of the core subunits  $\alpha$  or  $\epsilon$  results in a more stable complex with  $\beta_2$  while  $\alpha\Delta 7$  and  $\alpha_{wt}$  cores show similar binding to  $\beta_2$ . Positive ion nanoESI mass spectra were acquired as described in the legend to Figure 4, with mixtures of 2  $\mu\text{M}$   $\beta_2$  and various  $\alpha\epsilon\theta$  cores. **(A)** Cores assembled *in situ* from 0.9  $\mu\text{M}$   $\alpha\Delta 7$ ,  $\alpha_{wt}$ , or  $\alpha_L$  (as indicated), 2  $\mu\text{M}$   $\epsilon_Q$  and 5 mM  $\theta$ . The assembled cores contain the weakened  $\epsilon_Q$  CBM in  $\epsilon$ . **(B)** Cores assembled *in situ* from 0.9  $\mu\text{M}$   $\alpha\Delta 7$ ,  $\alpha_{wt}$ , or  $\alpha_L$  (as indicated), 2  $\mu\text{M}$   $\epsilon_L$  and 5 mM  $\theta$ . The assembled cores contain the strengthened  $\epsilon_L$  CBM in  $\epsilon$ .



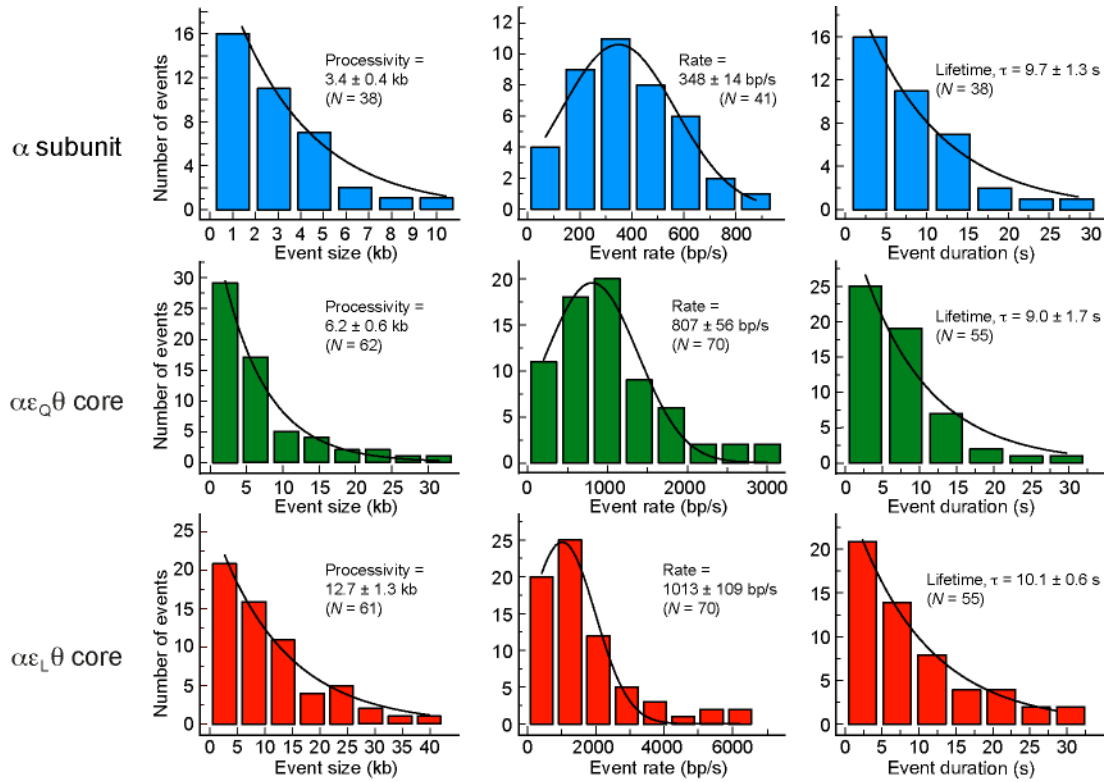
**Figure S5** Primer extension under "difficult conditions" strongly depends on both  $\beta_2$  and  $\epsilon$ , which does not have to be active as a proofreader. Primer extension assays (20 min) with 40 nM  $\gamma_3\delta\delta'$  and **(A)** 150 nM  $\alpha$  or isolated  $\alpha\epsilon\theta$  complexes in the absence and presence of 200 nM  $\beta_2$ , or **(B)** 150 nM  $\alpha$  or  $\alpha\epsilon$  complexes assembled *in situ* with 150 nM  $\alpha$  and 350 nM  $\epsilon_{wt}$ ,  $\epsilon_{D12A}$ ,  $\epsilon_{D12A,E14A}$  or  $ubq-\epsilon_{CTS}$ , in the presence of 200 nM  $\beta_2$ .



**Figure S6** Single-molecule (SM) leading strand replication assays. Representative SM replication trajectories. Examples of shortening traces including pauses (identified by dashed lines) during leading strand synthesis by wild-type Pol III HE assembled *in situ* as described in Supplementary methods. Note that all proteins are present continuously, and replication events on any single template DNA commence at random times during these experiments. This reflects the low efficiency of DnaC-dependent loading of DnaB in this situation where the template has no free 5' end and no replication origin. Thus DnaB must remain bound at the fork while another replisomal component dissociates during pausing.



**Figure S7** Single-molecule (SM) leading strand replication assays. Available (free) DnaC destabilizes DNA synthesis, reflected mostly in rate, but also processivity. Distribution of DNA synthesis rates (fit with a Gaussian distribution) and processivities (fit with a single exponential decay) for the overall number of events  $N$  during leading strand synthesis by Pol III HE in cases **(A)** of DnaB<sub>6</sub>(DnaC)<sub>6</sub> assembled *in situ* from approximately stoichiometric DnaB<sub>6</sub> (30 nM) and DnaC (180 nM) or **(B)** pre-isolated DnaB<sub>6</sub>(DnaC)<sub>6</sub> (standard conditions, 30 nM) supplemented with excess DnaC (90 nM).



**Figure S8** Single-molecule (SM) leading strand replication assays. The  $\epsilon$ - $\beta$  interaction affects both DNA polymerization processivity (left panels) and rate (center) but not duration of replication events (right), consistent with its role in stabilizing the replicase in the polymerization mode of processive DNA synthesis. Distribution of DNA synthesis rates (fit with a Gaussian distribution), processivities and event durations (fit with a single exponential decay) for the overall number of events  $N$  during leading strand synthesis by Pol III HE assembled in situ with  $\alpha$  alone, or isolated  $\alpha\epsilon_Q\theta$  or  $\alpha\epsilon_L\theta$  cores (60 nM, standard condition).